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Reverse Mutations in the Fragile X Syndrome

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Three females were identified who have apparent reversal of fragile X premutations. Based on haplotype analysis of nearby markers, they were found to have inherited a fragile X chromosome from their premutation carrier mothers, and yet had normal size *FMR1* repeat alleles. The changes in repeat sizes from mother to daughter was 95 to 35 in the first, 145 to 43 in the second, and 82 to 33 in the third. In the first family, mutations of the nearby microsatellites *FRAXAC2* and DXS548 were also observed. In the other two, only mutations involving the *FMR1* repeats were found. We suggest differing mutational mechanisms such as gene conversion versus DNA replication slippage may underlie such reversions. We estimate that such revertants may occur among 1% or less of premutation carrier offspring. Our results indicate that women identified to be carriers by linkage should be retested by direct DNA analysis.

KEY WORDS: fragile X syndrome, DNA repetitive sequences, mutation

INTRODUCTION

The fragile X syndrome is the most common inherited form of mental retardation [Brown and Jenkins, 1992; Warren and Nelson, 1994]. Previously, the diagnosis of the syndrome was established by the cytogenetic demonstration of a fragile site at Xq27.3 (FRAXA) under conditions of folate deprivation. The cloning and characterization of the fragile X gene (*FMR1*) led to direct DNA testing and recognition that the mutation responsible for the syndrome generally involves expansion of a repeated trinucleotide (CGG)n sequence located within the gene's 5′ non-coding region [Verkerk et al., 1991; Fu et al., 1991; Rousseau et al., 1991]. The normal number of repeats varies from approximately 6 to 55 with the most common Caucasian allele size being 30 [Brown et al., 1993; Snow et al., 1993]. Normal male and female carriers of the mutation generally have from approximately 56 to 200 repeats [Fu et al., 1991; Brown et al., 1993; Snow et al., 1993]. Such individuals are now commonly referred to as carriers of a "premutation." The concept of a premutation with regard to fragile X was initially suggested by Sherman et al. [1985] and by Pembrey et al. [1985] in the context of attempting to model the apparent delayed mutations seen in fragile X families. Premutations are generally considered to have a risk to expand to the full mutation in the next generation or in the second generation in the case of a transmitting male and are usually ascertained in the context of a family study of a full mutation fragile X index case [Fu et al., 1991; Warren and Nelson, 1994]. Full mutations as found in affected individuals generally have more than ~200 repeats are usually associated with methylation of an upstream promoter region and have a lack of gene expression [Pierretti et al., 1991]. The risk of expansion from a premutation to a full mutation is dependent upon sex of the parent, the size of the repeat, and by lack of interruptions of the pure CGG repeat with AGGs that may increase stability [Eichler et al., 1994; Kunst and Warren, 1994; Snow et al., 1994; Hirst et al., 1994; Zhong et al., 1995]. No new fragile X mutations have been described [Smits et al., 1993]. Because of this low frequency of new mutations, the fragile X syndrome is atypical of other severe X-linked diseases with reduced genetic fitness where new mutation rates are generally high to replace the loss of disease alleles.

Previously, we noted a family in which a reverse fragile X mutation was apparent in a daughter [Zhong et al., 1993]. We have now identified two more such females. These three unrelated females inherited the same flanking fragile X chromosome markers as their affected male brothers. In each case they were initially given high risks of being carriers, based on linkage analysis. However, direct mutation testing has shown each to have two normal-sized *FMR1* alleles. Thus, these three women have alleles that represent apparent reverse fragile X mutations.

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MATERIALS AND METHODS

Blood specimens were collected in EDTA tubes and DNA was extracted by phenol/chloroform or by using an extraction kit (Gentra, Inc). Testing for flanking RFLPs was done using probes and of microsatellite repeats by PCR using the referenced procedures. The closely flanking polymorphic loci that were typed included *DXS539* (pJH89) [van den Hurk et al., 1991], *DXS297* (VK23) [Suthers et al., 1991], *DXS548* [Riggins et al., 1992], *FRAXAC1* [Richards et al., 1991], FMRa [Kunst and Warren, 1994], *FRAXAC2* [Richards et al., 1991], *DXS465* (Do33) [Oberlé et al., 1991], FRAXE [Knight et al., 1993], *DXS296* (VK21) [Suthers et al., 1991], and *DXS374* (1A1) [Patterson et al., 1989]. The locations of the polymorphic loci used for haplotyping are indicated in Figure 1. Direct Southern blot analysis of the *FMR1* locus following digestions with enzymes EcoRI and EagI used probe StB12.3 following the method of Rousseau et al. [1991]. *FMR1* CGG repeat numbers were determined using our PCR method employing forward primer 1 and reverse primer 3 as previously described [Brown et al., 1993]. Cytogenetic procedures for fragile X detection were performed as previously described [Jenkins et al., 1992].

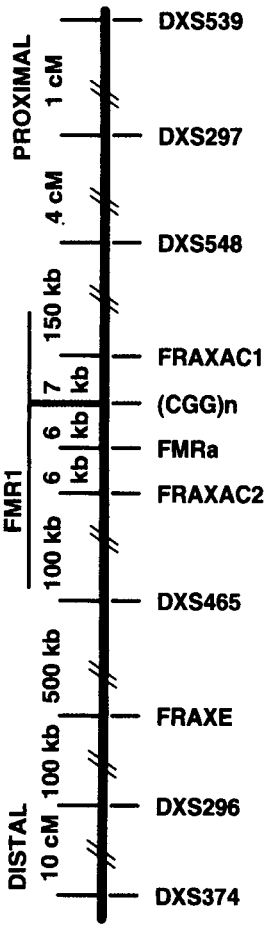

Fig. 1. Approximate location of DNA markers used relative to the *FMR1* gene and CGG repeat on the X chromosome.

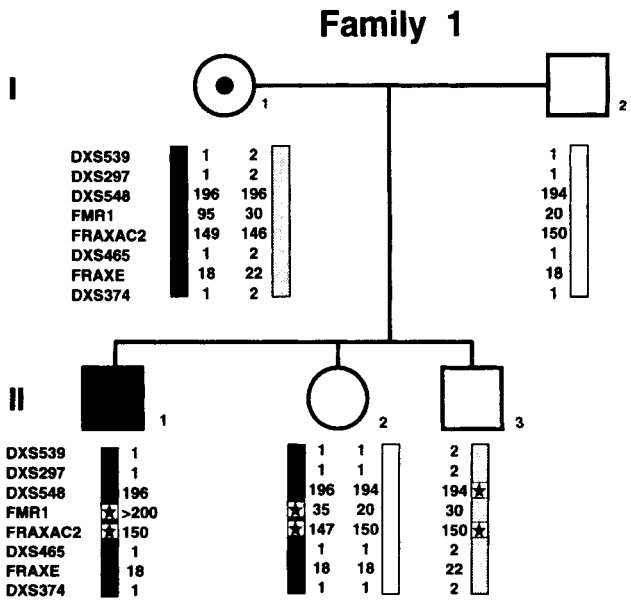

Fig. 2. Family 1 with DNA haplotypes. For Figures 2–4, the fragile X chromosomes are indicated in black, the maternal non-fragile X in gray and the paternal chromosome in white. The location of the reverse mutations of *FMR1* CGG repeats and of microsatellite mutations are indicated by stars.

RESULTS

The three family pedigrees and the informative markers studied are illustrated in Figures 2–4. *FMR1* allele PCR analysis is shown in Figure 5. Southern blot analysis using probe StB12.3 confirmed that the affected males had typical full mutations, that their mothers were premutation carriers, and that none of the three daughters showed a premutation pattern. Cytogenetic testing confirmed that at least one affected male in each family was positive for the fragile X chromosome.

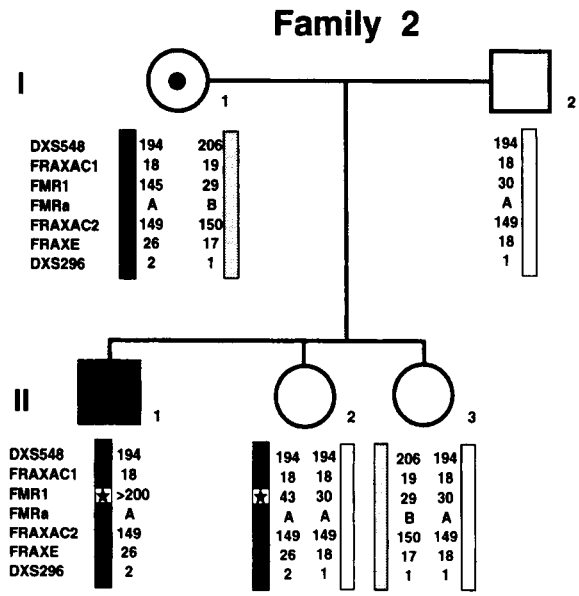

Fig. 3. Family 2 with DNA haplotypes.

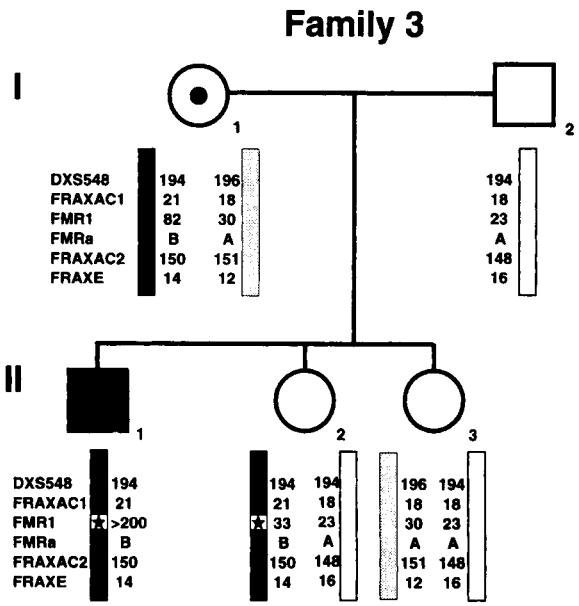

Fig. 4. Family 3 with DNA haplotypes.

Family 1

The first family included the carrier mother, I-1, with *FMR1* alleles of 30 and 95 repeats, the father, I-2, with 20 repeats, one affected son, II-1, with a full mutation, one normal daughter, II-2, with 20 and 35 repeats, and one normal son, II-3, with 30 repeats (Fig. 2). The daughter had three normal children (not shown), all of whom had inherited her 20 repeat allele. The daughter had inherited the same set of flanking markers from her carrier mother as that of her affected brother, including *FRAXAC1* and *DXS465*. Thus, she had inherited the fragile X region from her carrier mother and the mother's premutation allele had decreased from 95 to 35 repeats in the daughter. Previously, we briefly noted this family and that mutations were also observed in *FRAXAC2* [Zhong et al., 1993]. The carrier mother had *FRAXAC2* allele lengths of 146 and 149 bp. Her affected and her normal sons both had 150 bp alleles, while the daughter had inherited a 147 bp allele [Zhong et al., 1993]. Sequencing of *FRAXAC2* for the two brothers showed both had the same allele (X-16, Y-7, Z-14). In addition, the normal brother also had a 194 bp *DXS548* allele while the mother was homozygous for 196 indicating three loci showed mutations in this family. Repeat samplings on this family confirmed the typings were correct.

Family 2

The second family included a carrier mother, I-1, with *FMR1* alleles of 29 and 145 repeats, a father, I-2, with 30 repeats, one affected son, II-1, with a full mutation, one daughter, II-2, with 30 and 43 repeats, and a second normal daughter, II-3, with 29 and 30 repeats

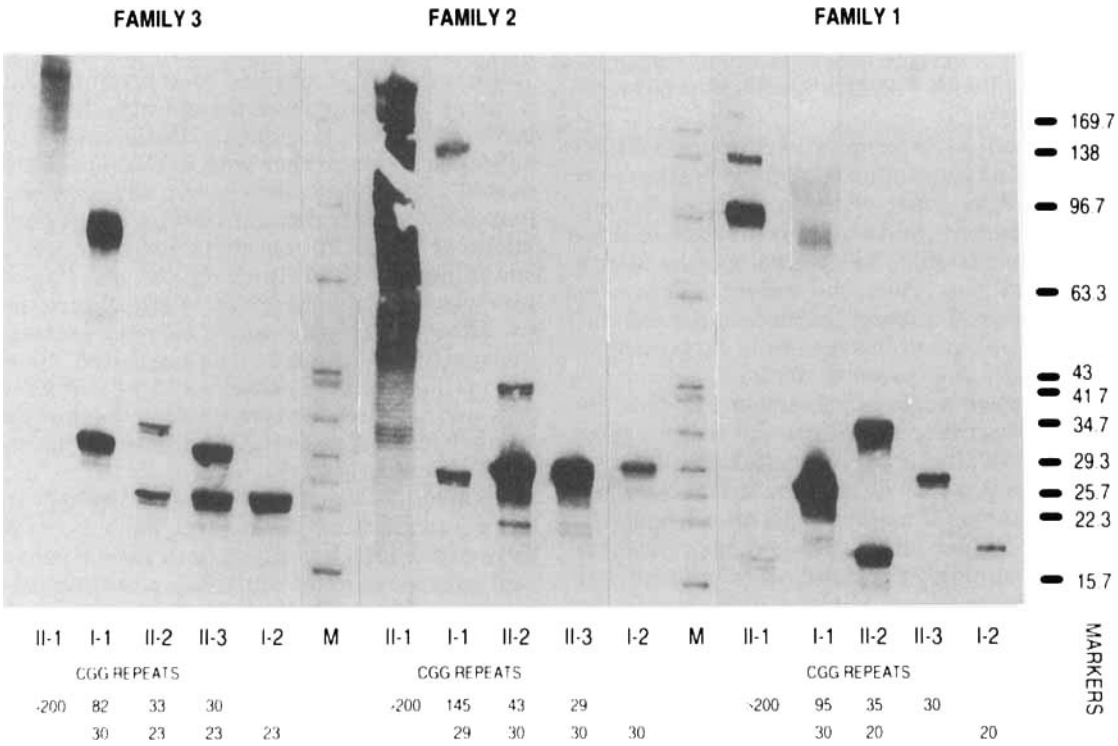

Fig. 5. PCR analysis of repeat sizes for the three families showing reverse mutations for the daughters. Pedigree numbers and lanes correspond to Figures 2–4. The daughters with reverse mutations are each indicated as II-2. All samples were analyzed on the same autoradiogram with a 1 hour exposure. Overnight exposure was required to better visualize the marker lanes (M) and some alleles (family 2: I-1, I-2; family 3, I-1).

(Fig. 3). Based on six informative flanking markers, the daughter, II-2, was found to have inherited the same fragile X chromosome as her affected brother indicating the mother's 145 repeat allele had undergone a reduction to the 43 repeat allele during transmission.

Family 3

The third family included a carrier mother, I-1, with *FMR1* alleles of 30 and 82 repeats, a father, I-2, with 23 repeats, one affected son, II-1, with a full mutation, one daughter, II-2, with 23 and 33 repeats, and a second daughter, II-3, with 23 and 30 repeats (Fig. 4). The carrier mother was informative for the two closest flanking markers, *FRAXAC1* and FMRa, as well as *DXS548* and *FRAXAC2*. These markers indicated that daughter II-2 had inherited the same fragile X chromosome as her affected brother but that she had a 33 repeat allele instead of an 82 repeat allele or a larger size indicating a reduction in length had occurred. We obtained a buccal epithelial sample for this daughter and typing confirmed she had allele sizes of 23 and 33.

DISCUSSION

These three examples of apparent reverse mutations are the only we have identified having analyzed the offspring of approximately 300 fragile X female premutation carriers. These events are not common and we estimate they may occur among 1% or less of meioses. These three women have normal-size but potentially unstable alleles and prenatal diagnosis may be advisable for their future pregnancies. Our results suggest that women who were previously counseled to have a high risk of being a carrier based on linkage analysis should be retested with *FMR1* mutational analysis to confirm their status.

Detection of such reverse mutations would be likely only if previous linkage studies and direct testing were discrepant or if PCR sizing analysis revealed discrepancies between parents and child. Since none of these daughters in these families has yet passed the altered repeat to the next generation, the stability of these alleles is yet unknown. The buccal sample in the one case tested was identical to her blood sample suggesting no somatic mosaicism was present. While mosaicism in which a premutation allele is present in the germline can not yet be disproven, we believe the risk for these women to bear a child with the full mutation is likely to be greatly reduced, since no full mutation child has been identified having a mother with an allele size of <55 repeats (J. Holden, these proceedings). We have found that approximately two thirds of premutation alleles lack any interrupting AGGs and the remainder have only one, and thus these three women are most likely to have a pure CGG repeats of 33, 35, and 43 [Zhong et al., 1995]. We lack confirmation of this conclusion, since we have not yet been able to adopt our method for AGG analysis to females. Further, cloning and subsequent sequencing, although possible, may be unreliable because of rearrangement artifacts. Instabilities of allele transmissions within the range of 36 to 55 repeats have been described indicating there exists a "gray zone" of potentially unstable alleles [Eichler et al., 1994, Zhong et al., 1995, 1996 these proceedings]. Which gray zone alleles should be considered as "premutations" in the absence of an identified close relative with a full mutation is unclear.

These events did not appear to involve a deletion of regions flanking the repeat, since the mutated alleles could be amplified by PCR primers that closely flank the CGG repeat region (ending 16 bp upstream and 5 bp downstream). Rather, the mutational change in each case appeared to be within the CGG repeat. In family one, both the CGG repeat and the *FRAXAC2* locus underwent mutational changes in two siblings. Since these loci are only separated by ~12 kb, the occurrence of related single events, such as gene conversion, seems likely. (The only known intervening polymorphism, FMRa, was uninformative, and thus we can not determine whether an unequal recombination event or whether two independent mutations occurred). Two microsatellite mutations involving *DXS548* and *FRAXAC2* were also observed in the normal male sibling in this family, but the *FMR1* allele remained unchanged, suggesting two double recombinations or a complex gene conversion. The reverse mutations in the second and third cases appeared to be confined to a change within the CGG repeat, as both of the closest upstream and downstream polymorphisms, *FRAXAC1* and FMRa, were the same as their affected brothers. Since there was no apparent recombination or mutation of flanking markers, the latter could be due to a simpler mechanism such as DNA slippage.

Three other instances of apparent fragile X reverse mutations have been reported. In the first [Snow et al., 1993], a daughter who had been given a high risk to be a carrier by linkage was found by Southern analysis to have two normal size alleles. In the second [Vits et al., 1994], a carrier mother with a 110 repeat allele transmitted a 44 repeat allele to her daughter. In the third [van den Ouweland et al., 1994], a carrier mother with alleles of 80 and 29 repeats transmitted a 29 repeat allele to her daughter. In the latter, proximal and distal markers with one exception (FMRb) were the same as the affected haplotype and a complex mutation involving multiple exchanges was suggested. Since each of the six identified revertants have occurred in daughters and no such revertants have been described yet among sons, a sex-related difference in mutational mechanisms may exist.

Gene conversion events appear to be very common at some human minisatellite loci, such as at MS32 [Jeffreys et al., 1994], in which both simple conversions as well as more complex multistep mutation processes involving allele reduplication plus inter-allelic conversion events have been identified. Gene conversion events between similar sequences may also be common, as is suggested by a high frequency of conversions between unlinked, hemizygous transgenes in the germline of mice [Murti et al., 1994]. Similar to fragile X, there have been several examples of reverse mutations described for myotonic dystrophy (DM). Analysis of one indicated a complex gene conversion event

[O'Hoy et al., 1993]. In two others the flanking markers were unchanged [Brunner et al., 1994], suggesting slippage.

There appears to be a size threshold of around 40 pure CGG triplet repeats above which *FMR1* allele instability is much more likely. As we first suggested [Brown et al., 1993], this threshold may correspond to the upper size limit of Okazaki fragments which could increase the probability of DNA slippage. This hypothesis has been further discussed [Kunst and Warren, 1994; Eichler et al., 1994; Richards and Sutherland, 1994]. It has also been noted that 40 repeats is the approximate threshold for the formation of stable hairpin loops which could promote expansion [Chen et al., 1995; Gacy et al., 1995]. Complex internal mechanisms that involve multistep processes resulting in rearrangement of flanking markers also could underlie such mutations [Jeffreys et al., 1994]. Analysis of additional such revertants may shed light on the molecular mechanisms which underlie triplet repeat instability.

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